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13. ABSTRACT *(Maximum 200 words)* Autocrine motility factor (AMF) is a 125kDa dimeric protein with two distinct functions: Intracellularly, it is an essential, ubiquitously expressed glycolytic enzyme. Extracellularly, it is also found in the circulation of patients with cancer, particularly with advanced, metastatic disease. Release of the extracellular form is by an unknown mechanism, but the factor binds to a known receptor with high affinity and has a number of effects, including stimulation of cell motility and differentiation of monocytic cells. We show here that AMF is a potent stimulator of the formation of osteoclasts, cells which resorb bone. The effect is species-specific by about 100X between mouse and human AMFs and their receptors. The dose-response is bell-shaped with maximum effects at 1ng/ml. In vitro mouse AMF induces RANK ligand expression in a bone marrow stromal line. RANK ligand is the central regulator of osteoclast formation. Cells secreting mouse AMF formed tumors in the muscles of nude mice and caused several dramatic effects compared to equal-sized control tumors: 1) Systemically, AMF secretion caused severe weight loss (cachexia). 2) Locally, AMF stimulated formation of disorganized new bone between the tumor and the surface of the femur. The data suggest that AMF is a novel stimulator of local changes in bone during metastasis and may also be a systemic mediator of cachexia, a serious complication of neoplasia.

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FOREWORD

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John M. Arrigan 4/29/99

PI - Signature Date

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Role of Autocrine Motility Factor in Osteolytic Metastasis

FIRST YEAR PROGRESS REPORT

INTRODUCTION:

Autocrine motility factor (AMF) is expressed by a wide variety of tumor cells, including several melanoma, fibrosarcoma, and prostate cancer and human MCF7 breast cancer cells (Silletti & Raz, 1996). It increases invasiveness through extracellular matrix and cell motility by a number of tumor lines. AMF was recently purified and its sequence analyzed (Watanabe et al, 1996; Niinaka et al, 1998); it was found to be identical to a previously characterized molecule, neuroleukin (Gurney et al, 1986), which is secreted by stimulated lymphocytes and is the extracellular form of phosphoglucose isomerase (Chaput et al, 1988), a glycolytic enzyme. The pathway of secretion of AMF is unknown but may follow a nonclassical route (Kuchler, 1993), similar to that taken out of cells by FGFs 1 & 2 (Florkiewicz et al, 1995). Serum phosphoglucose isomerase levels were used as a marker of metastatic breast disease over 40 years ago and found to correlate with the growth of tumor in bone (Bodansky, 1954). Since AMF is secreted by multiple tumor types by an unknown mechanism, it has not been used widely as a prognostic marker (Baumann et al, 1990).

The cloned autocrine motility factor receptor (Watanabe et al, 1991) is a commonly expressed membrane protein with sequence homology to p53 (Silletti & Raz, 1996). AMF binding to its receptor stimulates signaling through protein kinase C. The effects of AMF are duplicated by the 12-lipoxygenase product, 12(S)-HETE, which stimulates tumor cell invasiveness and motility *in vitro* (Honn et al, 1994). Like AMF and other motility factors (Liotta & Kohn, 1990; Stoker & Gherardi, 1991), 12-lipoxygenase activity is elevated in highly metastatic tumor line variants. Cell motility and invasiveness play complex roles in the metastasis of many tumors (Nicholson, 1988), and AMF and its receptor contribute to these processes. However, it is unclear why certain tumors commonly metastasize to specific sites (Nicholson, 1988; Rusciano & Burger, 1992), such as bone in the case of breast cancer.

Breast cancer metastasizes to the skeleton more frequently than any other tumor type, causing devastating complications. Over 70% of patients dying from breast cancer have metastases to bone (Coleman & Rubens, 1987). These are more commonly osteolytic than osteoblastic (Guise & Mundy, 1995) and cause bone pain, pathologic fracture, hypercalcemia and nerve compression syndromes (Aaron, 1994). Breast cancer displays osteotropism - a pronounced affinity to grow in bone (Guise & Mundy, 1995), which has been recognized for over a century (Paget, 1889). Bone matrix stores large quantities of immobilized growth regulatory factors (such as TGF-β, IGFs 1 & 2, FGFs 1 & 2, PDGF, and BMPs) which are released and activated during osteoclastic bone resorption (Hauschka et al, 1986; Mohan & Baylink, 1991). These factors can regulate the growth of tumor cells, such as those of the breast, which very commonly form osteolytic metastasis. If breast cancer cells produce factors which stimulate osteoclastic bone resorption, then metastasis to bone will be enhanced by a paracrine loop: tumor cell

products stimulate osteoclasts; products released from the resorbed bone matrix in turn stimulate the growth of tumor cells. Tumor-secreted factors capable of stimulating bone resorption are thus prime candidates to contribute to the establishment of osteolytic metastasis (Yoneda, 1996). The factor best characterized in this regard is parathyroid hormone-related protein (PTHrP) (Philbrick et al, 1996), which is commonly secreted by breast cancer, is prognostic of metastasis, and known to stimulate bone resorption (Guise & Mundy, 1996, 1998; Bundred et al, 1996; Guise et al, 1992). PTHrP is expressed by 90% of breast cancer metastases in bone. At high levels in the systemic circulation, it is the major causative agent of humoral hypercalcemia of malignancy. PTHrP is one of several factors known to stimulate osteoclastic bone resorption (Roodman, 1996), including calcitriol, interleukins 1 & 6, M-CSF, TGF-α, and TNF-α, to which list we have now added AMF. Although PTHrP is probably the primary factor responsible for osteolytic metastases by breast cancer, other tumor-secreted factors certainly play critical roles, by acting at different steps in the differentiation of bone-resorbing osteoclasts. Our group has shown, for example, that interleukin-6 enhances the resorptive effects of PTHrP *in vivo* (De La Mata et al, 1995). Although AMF is secreted by tumors which do not commonly metastasize to bone, we believe it may play a special role in breast cancer by acting in concert with PTHrP (and possibly other tumor-secreted factors) to stimulate bone metastases.The vicious cycle whereby tumor cells and osteoclasts stimulate one another can be interrupted by bisphosphonate inhibitors of bone resorption (Sasaki et al, 1995; Yoneda et al, 1997; Fleisch, 1991) or blocking antibody (Guise et al, 1997) against PTHrP. These treatments do not eliminate metastases, and bisphosphonates are palliative without increasing patient survival (Siris, 1997; Hortobagyi et al, 1996). Thus, it is essential that the roles of addtional factors in bone metastasis be elucidated. Factors which control cell adhesiveness play important roles in tumor metastasis, and antagonists of specific adhesive interactions decrease bone metastases, as does inhibition of tumor-expressed matrix metalloproteinase. We have recently shown that altering PTHrP secretion from MDA-MB-231 cells, by changing the responsiveness of the cells to TGF-β, correspondingly alters the ability of the cells to metastasize to bone (Yin et al, 1999). These experiments demonstrate that the cardiac ventricle injection/nude mouse model can be used to quantify changes in metastasis in response to changes in the stimulation of osteoclastic bone resorption.

BODY OF REPORT

METHODS:

General Methods. Cell line are obtained from the ATCC and maintained by standard means. RNA is isolated with kits from BRL and RT substrates for PCR were converted from RNA with oligo(dT) primer andthen amplified with Superscript II Taq polymerase with kits, also from BRL. PCR primers were designed using MacVector software and sequence files obtained online fromthe Entrez database. Primers were synthesized locally and PCR was carried out in a MJ cycler. Products were analyze by agarose gel electrophoresis, ethidium Br staining, and photography. Most reagents were from Sigma.

AMF is secreted breast cancer cells and regulated by factors released from bone matrix. There is little published information on AMF secretion by breast cancer lines or on its regulation by growth factors, including those released into the bone microenvironment during osteoclastic bone resorption. A series of standard human breast cancer cell lines (5 ER+: MCF7 [known to make AMF], MDA-MB-361, T47D, BT483, ZR75-1, and 5 ER-: MDA-MB-231 [which we have found to make AMF], -435, -436, BT549, Hs578T) were grown and used to condition serum-free media for 48 hours. All of these line cause metastases in the mouse model, with a slower time course than MDA-MB-231 cells (Yin & Guise, unpublished). Cell number and protein concentration was determined and AMF assayed via the coupled spectrophotometric assay for phosphoglucose isomerase (Noltmann, 1966). [Conversion of fructose 6-P to glucose 6-P is followed with glucose 6-P dehydrogenase and increase of A_{340nm} due to reduction of NAD.] Secreted AMF was corrected for cell number. Cell lysis was determined by assaying for the cytoplasmic marker lactate dehydrogenase, since release of cytoplasmic AMF can occur by lysis, in addition to secretion. LDH was assayed with a commercial kit (Sigma).

Mouse AMF was purified from serum-free conditioned media of CHO cells stably transfected with mouse AMF cDNA [ATCC, CHO-IC6], which make 40ng/ml/24hr/10^6 cells, and from human red cell hemolysate by affinity binding to phosphocellulose and specific elution with glucose 6-phosphate (Sun et al, 1990). The two purified proteins were tested in the human and mouse long-term marrow assays (Takahashi et al, 1988; Udagawa et al, 1989; Roodman, 1996).

The CHO-1C6 cell line was generated by transfection with a mouse neuroleukin (=AMF) expression vector (Gurney et al, 1986) and then increasing the level of expression by stepwise gene amplification of a linked dhfr cassette with escalating amounts of methotrexate. Such a stepwise selection takes approximately one year. We have maintained the CHO-1C6 cell line in culture for >60d in the absence of antibiotic selection, at the end of which period the constitutive secretion of mouse AMF was stably maintained. CHO cells injected into nude mice grow in bone. Five mice per group were studied by intramuscular inoculation of 1 million cells into each thigh. Untransfected CHO cells secrete <0.5ng/ml/24hr/10^6 cells. Animals were sacrificed at 15 days. Two groups of 10 animals each were inoculated with 10^5 cells by the left ventricle injection model. Animals were sacrificed at 3 weeks because of weight loss. All these animals had bone lesions on x-ray.

Animal experiments. The number of animals (n) to be tested is calculated by power analysis (probability of type I error α=0.05; probability of type II error β=0.20) based on previous data. We assay AMF in the peripheral circulation. PTHrP levels in the peripheral circulation are often not elevated into the detectable range in the presence of osteolytic metastases clearly visible on x-ray Enhanced osteolysis can, however, result in hypercalcemia; so we will monitor calcium. No changes in whole blood ionized calcium have been found in the experiments to date (not shown). Blood will be obtained by supra-orbital puncture from mice under anesthesia. Statistical analysis will be performed using analysis of variance followed by Tukey-Kramer multiple comparison's test on the SAS program for analysis of hypercalcemia, area of osteolytic lesions and metastases scoring. PTHrP will be measured by a commercial two-site IRMA assay (Nichols Institute) and AMF

by enzymatic assay. Whole blood ionized calcium concentrations will be measured using a Ciba Corning 634 ISE Ca^{++}/pH analyzer, which uses a sample volume of 35 µl. Samples are run in duplicate and the mean value recorded.

Bone histology and histomorphometry: Calvarial bones removed from mice are fixed in 10% buffered formalin. The posterior halves are decalcified in 14% EDTA and embedded in paraffin after processing. Thoraco-lumbar vertebrae, radiologically affected and unaffected forelimb and hindlimb long bones will be removed from mice at sacrifice, fixed in 10% buffered formalin and then decalcified in 14% EDTA. Sections will be cut using a standard microtome and placed on silane or poly-L-lysine-coated glass slides. Several consecutive sections will be cut at regular levels throughout the specimens. Adjacent sections at each level will be used for histomorphometry and stained using hematoxylin, eosin, orange G and phloxine. The following variables are measured in sections of bone to assess bone resorption: total bone area; total tumor area; total eroded and osteoclast surfaces (e.g., the extent of crenated surfaces and of crenated surfaces associated with osteoclasts, expressed respectively as a percentage of the total length of the interface between bone and bone marrow inside the calvariae.) Osteoclast numbers inside the calvariae are expressed per mm^2 of the total bone area.

Scoring of bone metastases: Animals are anesthetized deeply, laid down in a prone position against the films (22 x 27 cm X-O mat AR, Kodak) and exposed with an x-ray at 35 KVP for 6 seconds using a Faxitron Radiographic Inspection Unit (Model 8050-020, Field Emission Corporation, Inc.). Films are developed using a processor. All of the radiographs of the bones in nude mice are evaluated extensively and carefully by 3 different individuals in a blinded fashion. The number and area of osteolytic bone metastases will be calculated on radiographs using a computerized image analysis system. In this system, examination of osteolytic lesions from radiographs are achieved using a fluorescent light box (Kaiser, Germany) and Macro TV Zoom lens 18-108 mm f2.5 (Olympus, Japan) attached to a color video camera (Model DXC-151, Sony, Japan). Video images are captured using a frame grabber board (Taga+, Truevision, Inc., USA) installed in an IBM compatible 486/33MHz computer system. Quantitation of lesion area/number is performed using image analysis software (Java, Jandel Video Analysis, Jandel Scientific, CA, USA). When the radiographs are visually inspected, osteolytic metastatic foci as small as 1 mm in diameter are recognized as demarcated radiolucent lesions in the bones. The use of this image analysis system enables us to visualize lesions as small as 0.1 mm It is possible that micrometastases are present in the bones that will not be detected on the radiographic films. These will not be possible to quantify radiographically. However, detailed histologic analysis of the skeleton will be performed to identify such lesions.

Protocol for a typical osteolytic metastasis experiment: After baseline whole-body radiographs and blood for calcium measurement have been obtained, female athymic nude mice will be inoculated into the left cardiac ventricle with CHO cells or MDA-MB-231 cells. The latter consistently cause osteolytic lesions following left intracardiac inoculation with 10^5 tumor cells suspended in 100 µl of phosphate buffered saline (PBS). Based on

Dr. Guise's previous experience in this technique, performed on several hundred animals, about 90% of mice inoculated will develop osteolytic lesions. Cells are grown to confluence, trypsinized, washed twice with PBS and resuspended with PBS to a final concentration of 10^5 cells/100µl immediately prior to inoculation. Animals are deeply anesthetized with ketamine/xylazine and positioned ventral side up. The left cardiac ventricle is punctured through a percutaneous approach using a 27 gauge needle attached to a 1 ml syringe containing suspended tumor cells. Visualization of bright red blood entering the hub of the needle in a pulsatile fashion indicates correct position in the left cardiac ventricle. Tumor cells are then inoculated slowly over 1 minute. Two weeks following tumor inoculation, whole-body radiographs will be taken and animals in each group inspected for osteolytic lesions. Further radiographs will be obtained on a weekly basis thereafter to follow the progression of osteolytic lesions. Ca^{++} and body weight will be measured weekly for two weeks post tumor inoculation and then every three days thereafter for the remainder of the experiment. Animals will be sacrificed when paraplegic or severely cachectic, as our previous data suggest that body weight appears to be the most sensitive indicator of survival. At the time of sacrifice, blood will be collected for Ca^{++} measurement and all bones and soft tissues will be harvested in formalin for histologic analysis (6 tissue blocks per animal). Autopsy will be performed on all mice and those with tumor adjacent to the heart will be excluded from analysis, as this indicates that part of the initial tumor inoculum did not properly enter the left cardiac ventricle. It has been our experience that the adrenal is the secondary site for metastasis in the left ventricular injection model with MDA-MB-231 cells. Area of osteolytic lesions on radiographs will be quantified by a computerized image analysis system, after comparison with baseline radiographs. Careful attention will be paid to measurement of the size of the metastases. Osteolytic lesions will be confirmed histologically and tumor volume calculated by this method as well.

RESULTS

1) **AMF stimulates osteoclast formation**. Simultaneous with the identification of the AMF sequence, the same protein was independently purified and sequenced, using an assay for the differentiation of the monocytic HL-60 cell line (Xu et al, 1995). The differentiation of HL-60s has been used as a model of osteoclast formation (Roodman, 1996); so we reasoned that AMF could increase osteoclast number by stimulating their formation from precursors. We found that purified rabbit AMF stimulates the formation of multinucleated osteoclast-like cells in human long-term marrow culture assay. AMF was effective at 1ng/ml and capable of stimulatory effects equivalent to 10nM $1,25(OH)_2$vitamin D_3 (Fig. 1). [The first two panels of this Fig were included with the original submission of this proposal. All other data presented here are new.] It was without effect in organ culture assays of bone resorption (not shown), which test for stimulation of preformed osteoclasts, rather than their formation. AMF was stimulatory at 1ng/ml but inhibitory at >100ng/ml, a biphasic effect also seen in motility, neuronal survival, and HL-60 differentiation assays. Bone marrow cultures were stained with a rat monoclonal antibody against gp78, the AMF receptor. Mononuclear precursor and marrow stromal cells expressed the AMF receptor by antibody staining and immunofluorescence (not shown). When rabbit AMF was tested

in the mouse, as opposed to human, marrow culture assay, a very small, albeit statistically significant, effect was seen. These data suggested that the effects of AMF might be species-specific, a possibility not previously reported.

2) **Effects of AMF are species-specific**. To address this previously unasked question, we purified both mouse and human AMF proteins by conventional means (Noltmann, 1966; Sun et al, 1990). The mouse protein was isolated from serum-free medium conditioned by the CHO-1C6 cell line which secretes recombinant mouse AMF (Gurney et al, 1986; available from the ATCC). The human protein was isolated from human erythrocytes by affinity chromatography on phosphocellulose and elution with glucose 6-phosphate substrate (Sun et al, 1990). Mouse and human proteins were judged to be 60-80% pure judged by their specific phosphoglucose isomerase enzymatic activities compared to the homogeneous protein, which has SA = 800units/mg. The mouse protein showed only one major band at 63kDa on 12.5% denaturing, reducing polyacrylamide gel electrophoresis, followed by silver staining (not shown). These preparations were then used in conventional mouse (Takahashi et al, 1988) and human marrow culture assays (Roodman, 1996).

Figure 2 shows that mouse AMF stimulated the formation of mouse osteoclast-like cells in culture with a bell-shaped dose response and a maximal effect at 0.5 ng/ml. In contrast human AMF was effective at stimulating mouse osteoclast formation only at approximately 100 times higher doses (Figure 3). In the human marrow assays, formation of human osteoclast-like cells was stimulated by human AMF (Figure 5), although higher doses were required than for the mouse:mouse experiments of Figure 2. Again, the cross-species experiment (mouse AMF and human cells) required very high doses of factor to achieve a response (Figure 4). From these experiments we conclude that AMF is a potent stimulator of osteoclast formation in culture and that the stimulation is species-specific. This is unusual for cytokines and factors known to act on osteoclasts (Roodman, 1996). The species-specific effect appears to be about 100 fold, but further experiments with purified factors and cloned receptors will be needed to place this estimate on a firm molecular basis. No report of the mouse AMF receptor has been published. In order to continue this work, we are planning to prepare recombinant mouse and human AMFs.

3) **Recombinant mouse and human AMF expression**. We have identified full length cDNAs encoding mouse and human proteins, after requests for the published clones went unanswered. The cDNAs were subcloned into the mammalian expression vector pcDNA3 (Clontech) and tested by transient transfection into cos7 cells. Conditioned media from these cells showed dramatically elevated phosphoglucose isomerase activity compared to that obtained from cells transfected with empty vector. In order to be able to distinguish the recombinant from endogenous proteins, we are now recloning these cDNAs, via PCR mutagenesis, to add C-terminal His6 extensions. Dr. C Davies (Univ. of Brighton, U.K.) advises [personal communication] that the C-terminus of the protein is solvent-exposed in the as-yet unpublished crystal structure (Kugler et al, 1998); so the C-terminus is the appropriate place to attach this affinity tag. In the coming year we will use the His-tagged proteins for expression in E coli as 1.2kb Nde I to EcoR I inserts in pET5a as an improved

source for recombinant protein. E coli expresses a soluble isomerase of similar activity to the mammalian enzyme; so we expect to be able to obtain soluble expression of the recombinant AMFs. The His tags will also be used in the subsequent animal experiments to distinguish host-derived from tumor-derived plasma AMF.

4) **Breast cancer lines secrete AMF without cell lysis**. Figure 6 shows that several human breast cancer cell lines secrete AMF into serum-free conditioned media (upper panel). The lower panel shows that the profile is altered slightly when the results are corrected for cell lysis by normalizing to lactate dehydrogenase, the standard marker for cell lysis. This experiment corresponds to tasks 1-3 of the statement of work). Since it is unknown how the cells might respond in vivo in terms of AMF secretion, it is unclear what significance these in vitro results have. To address this uncertainty, we carried out an experiment not originally proposed.

5) **Plasma AMF levels are elevated in mice with advanced bone metastases**. We assayed 10ul samples of plasma from individual mice at baseline and sacrifice (28 days), which received on day 0 MDA-MB-231 human breast cancer cells by intracardiac inoculation. The details of this experiment (but not the AMF data presented here) have been published earlier this year (Yin et al, 1999). Five animals receiving control breast cancer cells developed cachexia and advanced osteolytic bone lesions (right side of figure). All of these animals had elevated (2.5-4X) concentrations of plasma AMF at sacrifice. On the right side, are the parallel results from 3 mice which had decreased tumor burden and weight loss consequent to genetic manipulation of the tumor cells to decrease osteolysis. None of these animals showed elevated AMF levels. The data strongly suggest that AMF could play a causal role in bone metastasis and perhaps in tumor cachexia. It is unclear in these experiments, whether the plasma AMF increases are host- or tumor-derived. This important question will be investigated in the coming year.

6) **Mechanism of AMF stimulation of osteoclast formation**. At the time of the submission of the proposal, the mechanisms controlling osteoclast formation and activity were highly controversial and relatively poorly understood (Roodman et al, 1996). This situation has changed radically with the discovery of a pair of effector molecules in the interim (Martin et al, 1998). Bone marrow stromal and osteoblastic cells express (in response to stimulators such as PTH, PTHrP, 1,25dihydroxyvitamin D3, IL6 family member cytokines, and others) a membrane anchored protein variously called RANK ligand, TRANCE, OPGL, and ODF, which activates NfkappaB signaling via the RANK receptor on osteoclast precursors of the monocyte/macrophage lineage (Yasuda et al, 1998). The activating effects of RANK ligand are opposed by the actions of a soluble ligand-binding protein, osteoprotegerin (Opg, OCIF, Simonet et al, 1997). In marrow stromal cells, the expression of RANKL and Opg appear to be reciprocally regulated. Based on this new information, we tested whether AMF might act on osteoclasts through the RANKL/Opg pathway. We treated the mouse bone marrow stromal cell line ST2 (Udagawa et al, 1989), with purified mouse AMF for 24hrs and isolated RNA using a commercial kit. RNA was converted to cDNA with oligo(dT) and a commercial RT kit. This material was subjected to PCR for 30 cycles with primers for mouse RANKL, Opg, or GAPDH (not shown). The

results (Figure 8) clearly indicate that signaling through the mouse AMF receptor on ST2 cells stimulates RANKL mRNA and depresses Opg mRNA, with a similar bell-shaped dose response to that seen for osteoclast formation in Figure 2. The data clearly point to a specific molecular mechanism for AMF action on bone cells: a direct one on cells in the osteoblastic lineage (e.g., ST2) and an indirect one via RANKL on cells in the osteoclast lineage.

7) **Osteoblastic effects of AMF on bone in vivo**. (Task 4 of statement of work). Two groups of 5 mice each were given bilateral tumors of the thigh by intramuscular inoculation of CHO tumor cells. Control animals received the standard CHO-K1 cell line; experimental animals received the CHO-1C6 cell line, described above, which secretes mouse AMF consequent to a gene-amplified mouse AMF expression DNA. The experiment was halted at 15 days, since the experimental animal group had become moribund. The animals were assessed by Faxitron x-ray and found to have osteoblastic new bone formation in the area between the tumor and the outer periosteal surface, indicated by arrows in Figure 9, middle and right. None of the control animals showed this response.

8) **Plasma AMF levels are elevated in mice with CHO-AMF tumors**. An experiment of the sort shown in Figure 7, was carried out with the mice described in the previous paragraph. As shown in Figure 10, the mice carrying CHO-1C6 (+AMF) tumors had statistically significant increases in plasma AMF concentrations at sacrifice compared to controls. Increases are comparable to those seen in Figure 7 for mice with MDA-MB-231 bone metastases.

9) **Cachexia is increased in mice with CHO-AMF tumors**. The mice in this experiment showed statistically significant weight loss in the CHO-1C6 (+AMF) group compared to the control CHO-K1-bearing group (Figure 11). The data suggest that AMF may contribute to tumor-induced cachexia. Cachexia is a major source of morbidity and mortality in patients with advanced cancers. Its causes remain remarkably poorly understood (Mattys & Billiau, 1997), and effective clinical treatments of this paraneoplastic syndrome are lacking. Cachexia may be a systemic effect of elevated AMF levels.

10) **AMF locally induces new bone formation**. We have just begun to analyze the bone histology of the mice in this experiment. The induction of new bone formation, indicated by x-ray (Figure 9), has been confirmed by H&E stained sections. Three of the mice show dramatic formation of disorganized new bone outside the femoral shaft. This is continuous with the existing bone, which shows no disorganization. TRAP staining indicates areas of osteoclasts on this new bone. The bone shows relatively normal vascularization and lacks evidence of inflammatory infiltrates. In one animal new bone formation was less progressed, and their appear to be an extensive number of osteoclasts at the tumor:periosteal interface. It is possible that a transient wave of osteolysis precedes the subsequent, more extensive new bone formation. By contrast, the control (CHO-K1-bearing) mice had no signs of new bone formation but did have modest osteolysis of the femoral periosteum. These data are still under analysis and are not shown. When complete, they will be presented in next year's report (task 5 of statement of work.)

11) **AMF overexpression alters bone metastases due to CHO cells**. We have begun the metastasis experiment with CHO-K1 versus CHO-1C6 cells (Task 4). The animals all developed bone metastases and are now being analyzed. On x-ray, the bone lesions are altered by AMF overexpression. However, CHO-K1 cells themselves caused aggressive osteolytic lesions, which have not been described in the literature. This result makes task 6 unneccessary. Instead, in task 11, we will construct MDA-MB-231 cell lines (which are known to cause osteolytic metastasis consequent to their production of PTHrP) with mouse and human AMFs. As described above, the mouse and human cDNAs are being modified to carry His6 tags to permit us to distinguish the introduced factors from the endogenous host and tumor factors. [This experiment will be done with a microscale NiNTA bead binding assay. The beads separate His6-tagged from endogenous AMF proteins, which are then quantified by the phosphoglucose isomerase activity assay. This assay is exquisitely sensitive and allows much easier and more accurate determination of AMF protein levels than could be obtained with the present antisera against phosphoglucose isomerase, which are of poor sensitivity and specificity.] The necessity of this step was not originally anticipated, since we were not aware of the species-specificity of AMF action or of the induction of plasma AMF in mice with bone metastases caused by MDA-MB-231 human breast cancer cells. Thus, the technical steps of tasks 10 and 11 have increased, and these two aims will now take longer.

12) **Regulation of AMF secretion**. With the recognition that human tumors may induce mouse AMF in animal experiments, and that human AMF produced by human tumors may have only weak effects on the mouse AMF receptor, we have limited our work on looking for factors which stimulate AMF secretion from human breast cancer cell lines in vitro (tasks 1 & 2). We have found no induction of AMF secretion by treatment of cells with TGFbeta1 (1-20ng/ml), which induces PTHrP secretion (Yin et al, 1999) or with 1-10ug/ml of factors which are reported (Gurney et al, 1986) to increase AMF production from lymphocytes cultures: concanavalin A, bacterial lipopolysaccharide, phytohemagglutinin, or pokeweed mitogen (data not shown). Should experiments planned for the coming year show that AMF is tumor-derived in the animal model, we will return to these experiments as originally proposed. We believe it advisable to de-emphasize this avenue at the moment in light of the lack of progress throughout the field of nonclassical secretion (Florkiewicz et al, 1995). It is possible that tumor necrosis is a major source of released AMF, in which case looking at the cytokine regulation of AMF secretion by dead cells would be foolish.

CONCLUSIONS

We have shown that AMF is a potent, species-specific factor with dramatic effects on bone. It potently stimulates the formation of osteoclasts in both mouse and human cultures, provided that the same-species factor is used in the experiments. We have found that this effect may in part be mediated through the recently recognized RANK ligand pathway. AMF activates RANK ligand expression (and depresses the mRNA for osteoprotegerin, the soluble inhibitor of RANK ligand) on mouse bone marrow stromal cells. In the mouse experiments maximal effects were seen at about 1ng/ml. Thus the

factor is very potent. All the effects seen in vitro show bell-shaped response curves; so that very high and low plasma concentrations of AMF may be ineffectual. We have revised our experimental plans to test both mouse and human AMF expression from human tumors for their effect on bone. We have also designed new experiments to test whether the elevations of plasma AMF seen in tumor-bearing animals are due to host or tumor secretion and whether the later is due to tumor necrosis, which will show a parallel increase in plasma lactate dehydrogenase activity.

The data obtained in vitro predicted that AMF overexpression in vivo would enhance osteolytic bone metastases. However, our results with intramuscular tumors suggest otherwise. Systemic elevation of AMF appears to be directly associated with cachexia. Cachexia is the most common universal paraneoplastic symptom of advanced cancers, being responsible for much of the morbidity and mortality of advanced disease (Matthys & Billau, 1997). Many cytokines, in particular tumor necrosis factor alpha have been implicated, but none has been found to be a central causal factor. AMF, which has been studied for 50 years as a marker of advanced disease and metastasis may prove to be a previously unrecognized causal factor of cachexia.

In addition to this unsuspected systemic effect of mouse AMF in experimental animals, we have found an unanticipated response in bone. Osteoblastic new bone formation occurs in about 15% of human breast cancer metastases and the majority of prostate cancer metastases to bone. It may be that tumor-stimulated AMF has local effects to stimulate cells of the osteoblast lineage and systemic effects involving cachexia. The interaction between AMF and its receptor gp78 remain essentially unstudied. This interaction appears to be species-specific, and this is probably unrelated to the phosphoglucose isomerase active site enzymatic activity (which is contributed by both monomer subunits at the dimer interface). Recent progress (Kugler et al, 1998; Davies, personal communication) suggests that the X-ray structure of the AMF protein will be completed soon. It may well be possible in the future to target the AMF:AMFR interaction to block the pathological effects of breast cancer associated AMF secretion. This interaction could be blocked without inhibiting the essential intracellular glycolytic function of the enzyme.

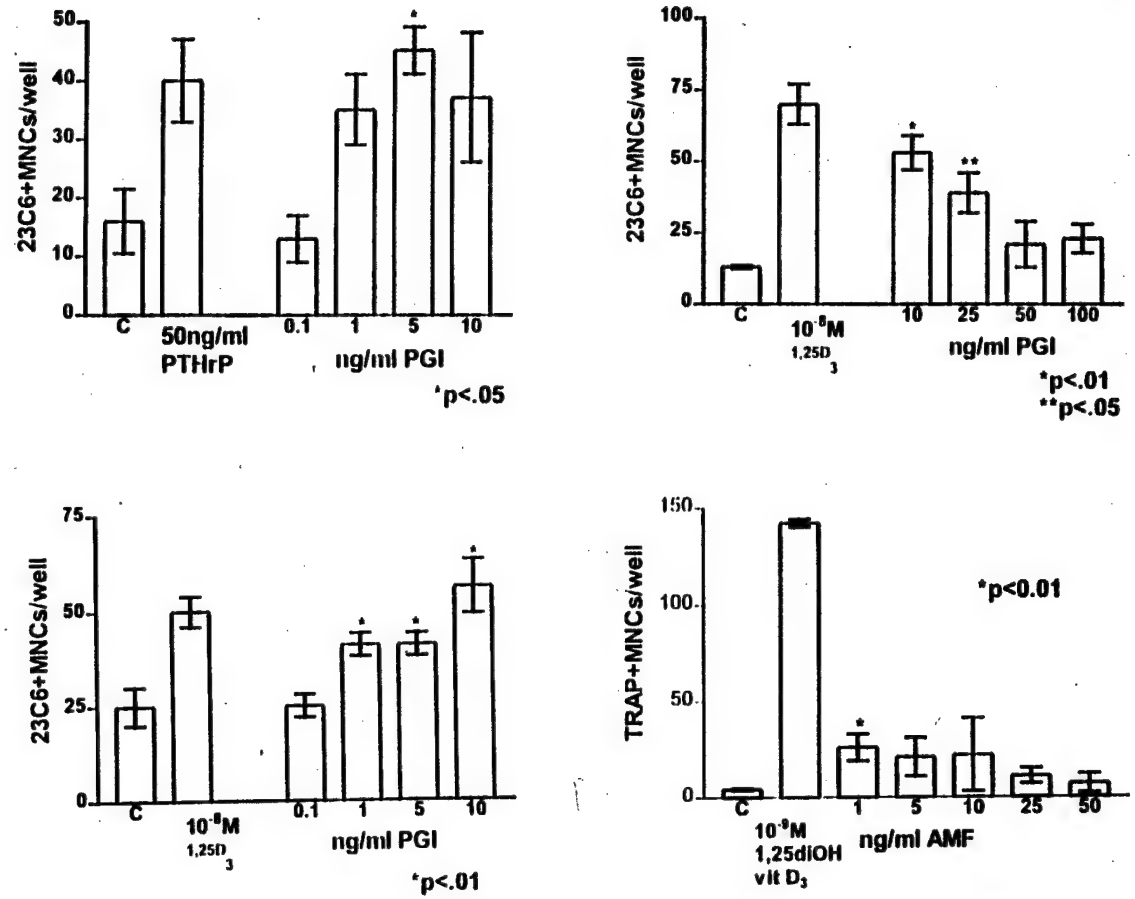

Figure 1. Human (1st 3 panels) and mouse (4th panel) marrow cultures were treated with rabbit AMF and scored for 23c6+ or TRAP+ MNCs, respectively. Rabbit AMF is believed to resemble the human factor.

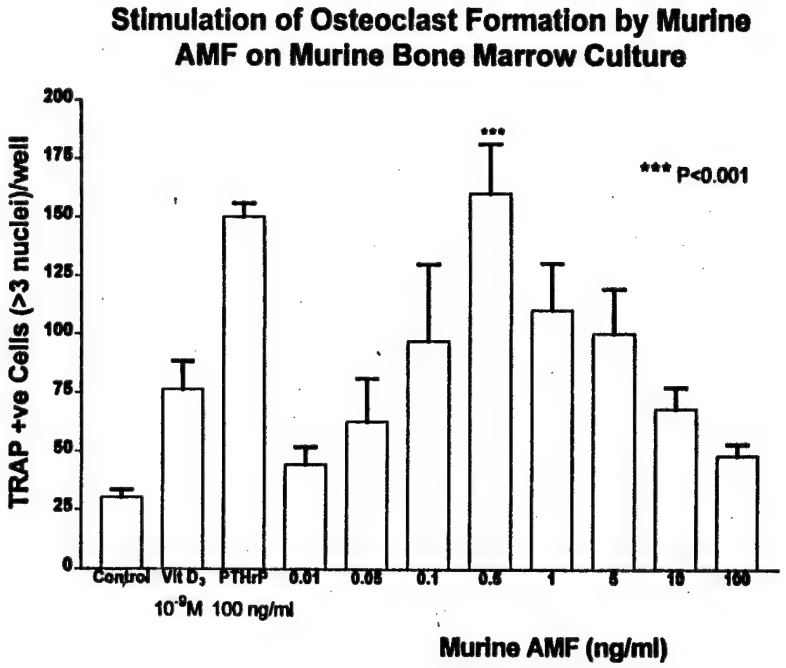

Figure 2. Mouse marrow cultures (7 days, in 48-well dishes) were treated with purified **mouse AMF** and scored for TRAP+ MNC formation (n=4).

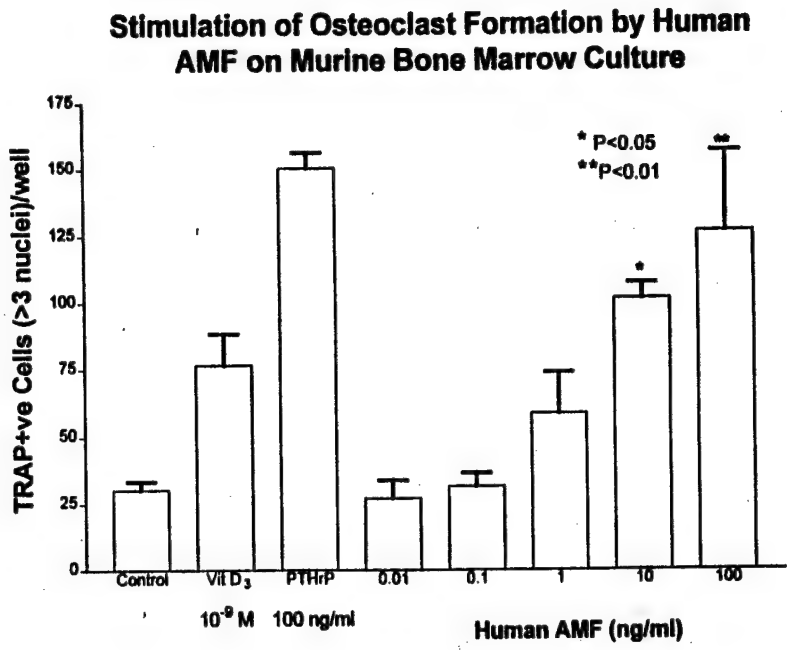

Figure 3. Mouse marrow cultures were treated with purified **human AMF** and scored for TRAP+ MNC formation (n=4).

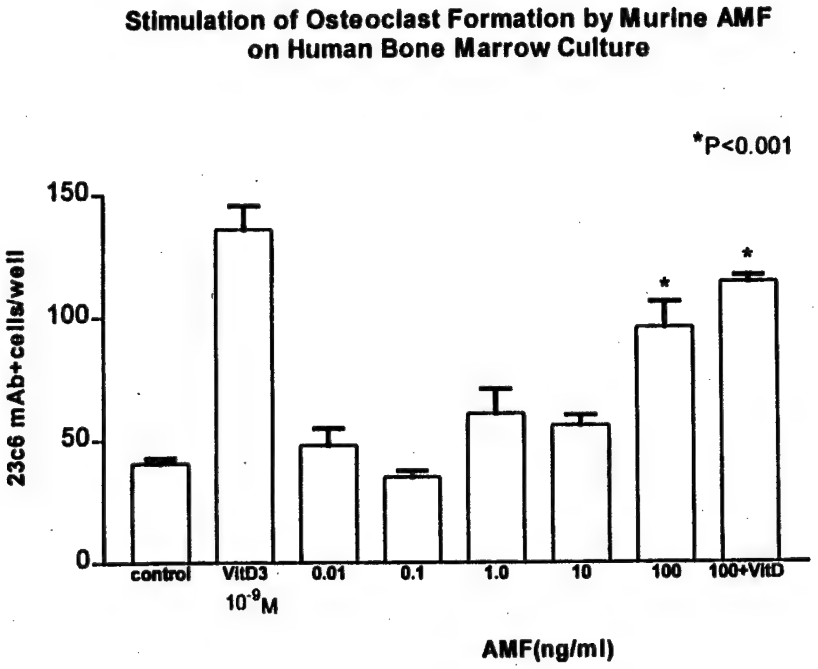

Figure 4. Human marrow cultures (21 days, in 96-well dishes) were treated with purified **mouse AMF** and scored for 23c6+ MNC formation (n=4).

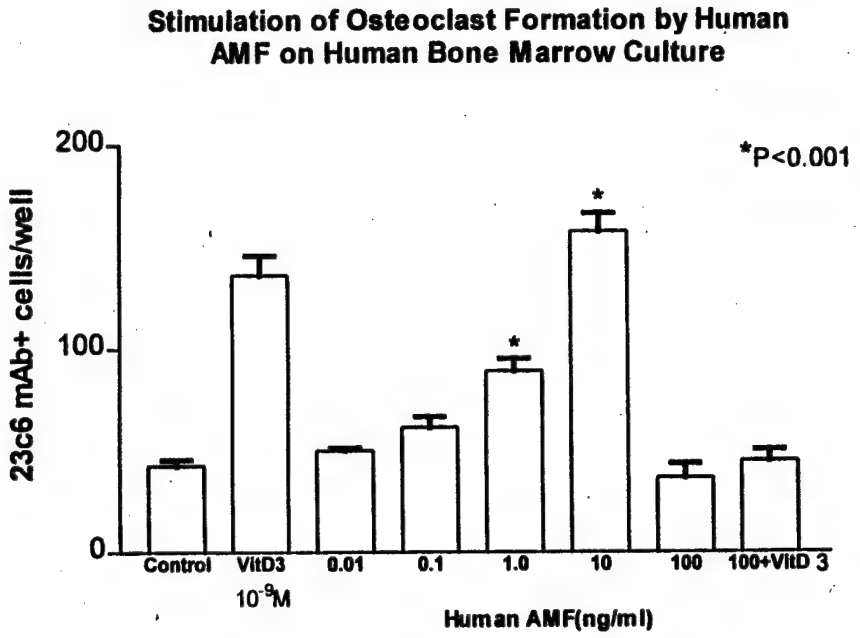

Figure 5. Human marrow cultures were treated with purified **human AMF** and scored for 23c6+ MNC formation (n=4).

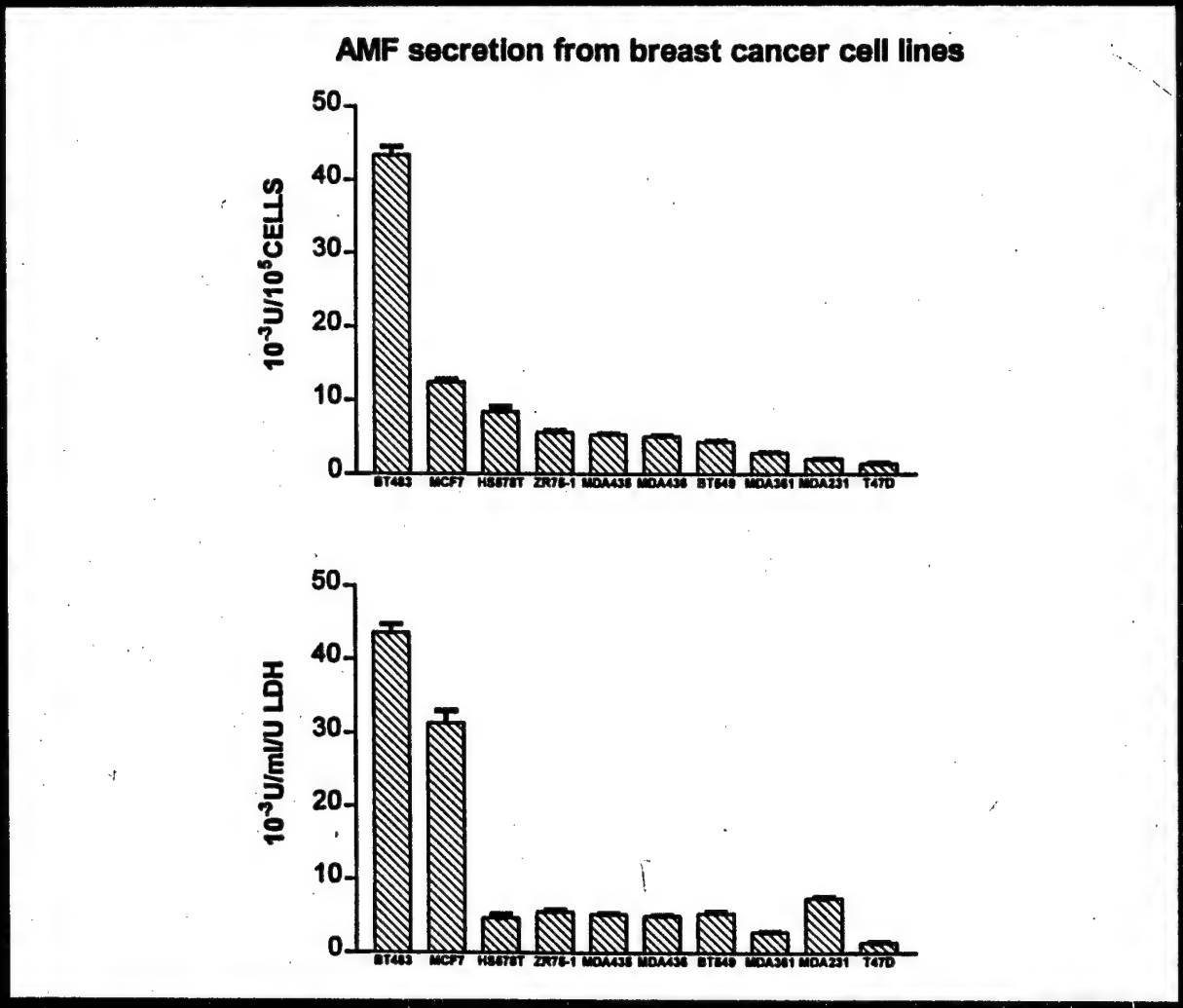

Figure 6. Human breast cancer cell lines were cultured to near confluence and placed in serum-free media for 48h. The isolated media were assayed for AMF by PGI enzymatic activity and for cell lysis by assay for the cytosolic marker enzyme lactate dehydrogenase (LDH), and cell numbers were counted.

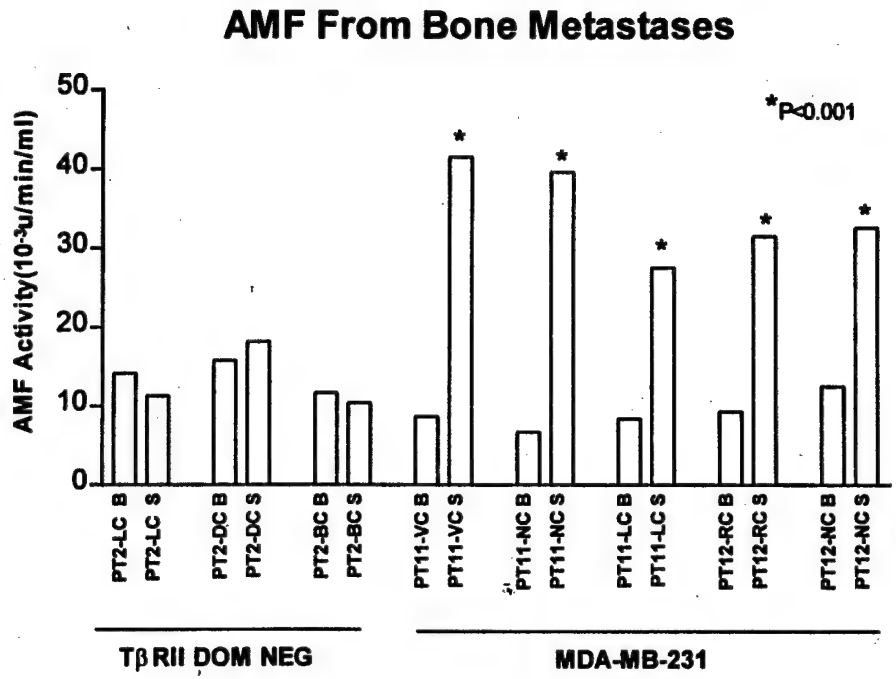

Figure 7. Plasma samples (10μl) obtained retro-orbitally from nude mice bearing MDA-MB-231 tumors metastatic to bone (Yin et al, 1999) were assayed retrospectively for AMF by PGI enzymatic activity. For each animal, **B** indicates baseline sample and **S** indicates sacrifice (28 days after intracardiac inoculation of breast cancer cells).

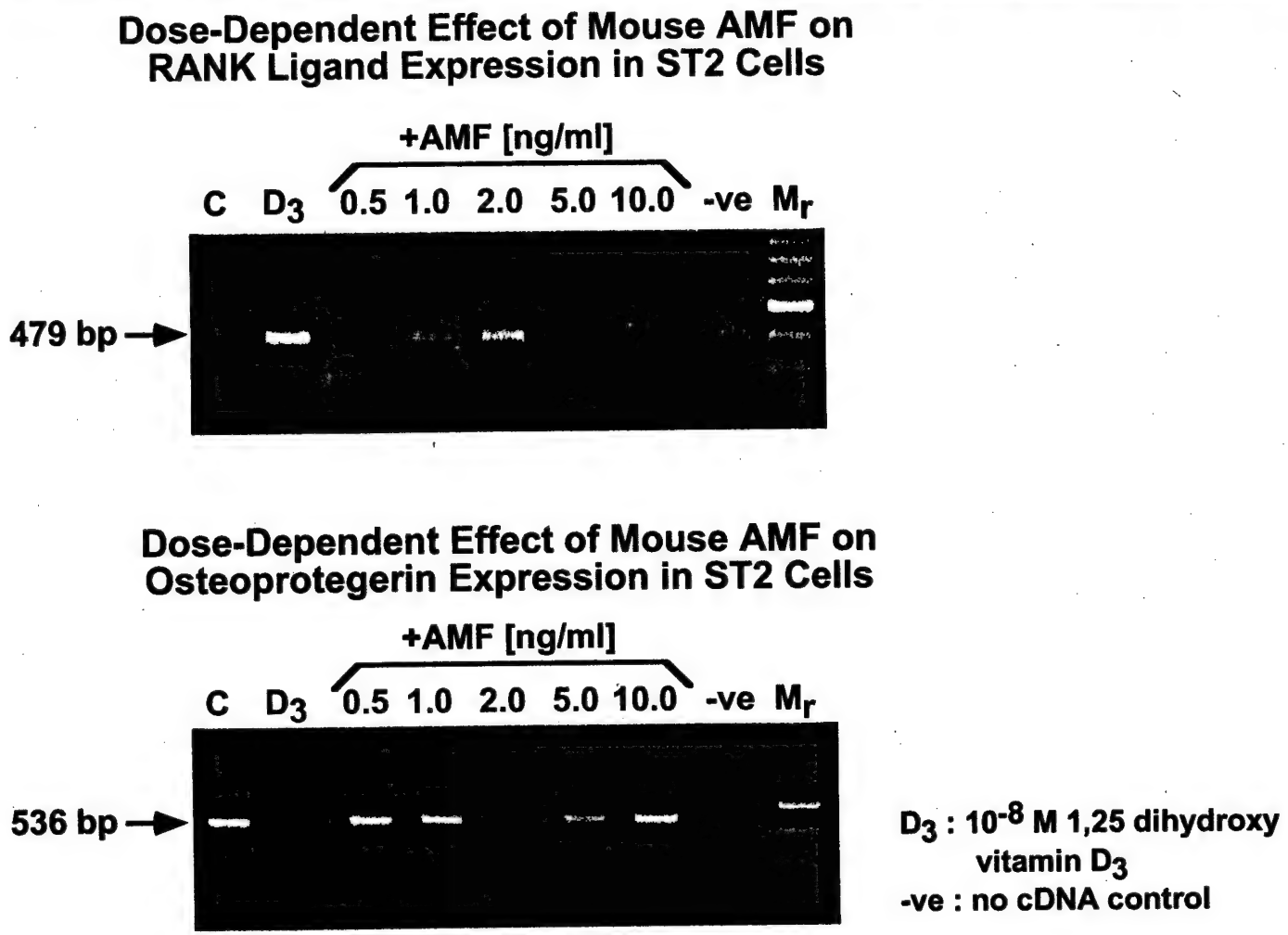

Figure 8. ST2 murine bone marrow stromal cells were cultured at confluence in the presence of varying concentrations of purified AMF or 1,25dihydoxyvitamin D3 as positive control. RNAs were isolated and reverse transcribed. The samples were analyzed by 30 cycles of PCR with mouse-specific primers for RANK ligand and osteoprotegerin kindly provided by Dr. B. Oyajobi.

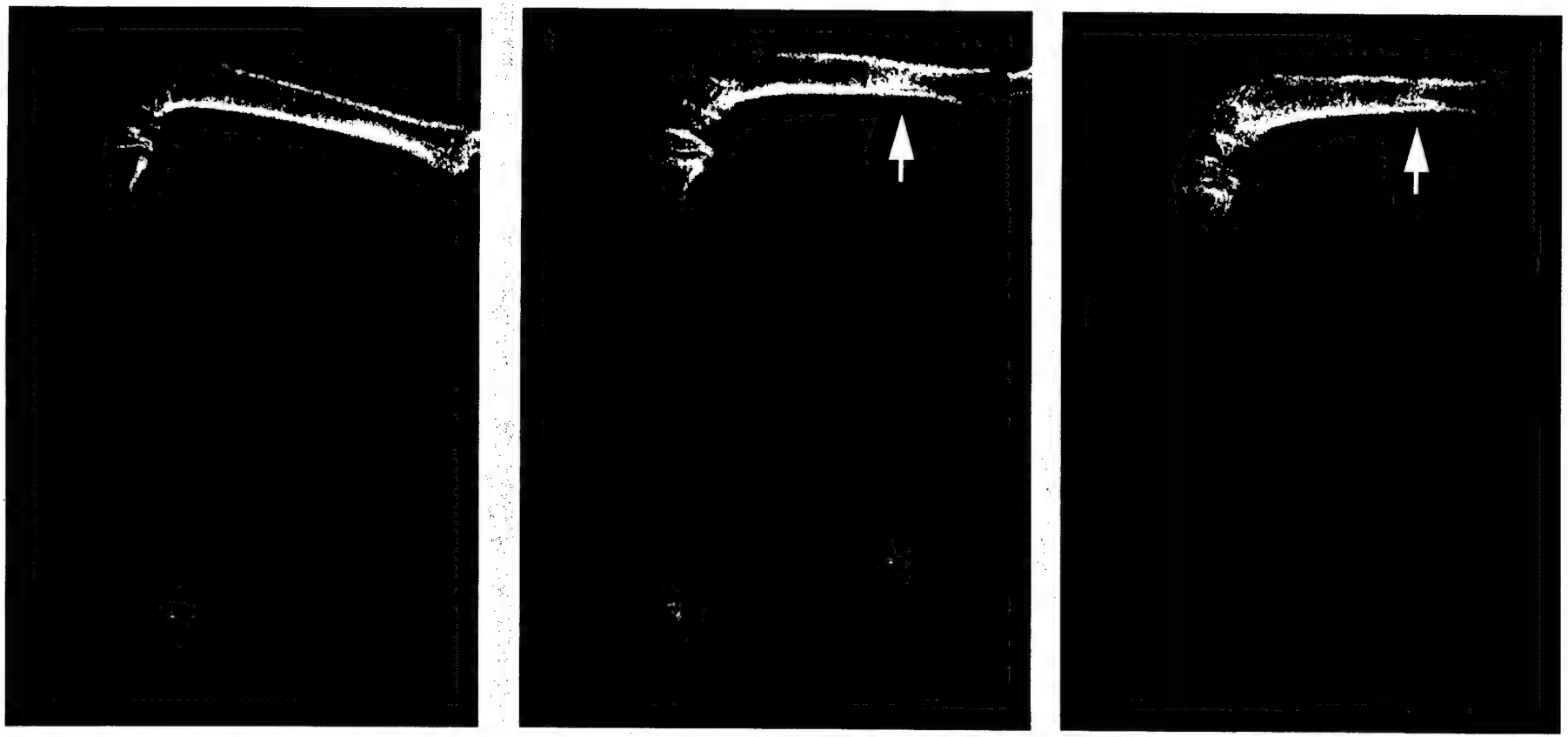

Figure 9. CHO-1C6 cells cause osteoblastic bone changes. Nude mice carrying CHO intramuscular tumors of equal size were x-rayed at 15 days (sacrifice) by Faxitron. Arrows indicate osteoblastic responses at the femoral surfaces.

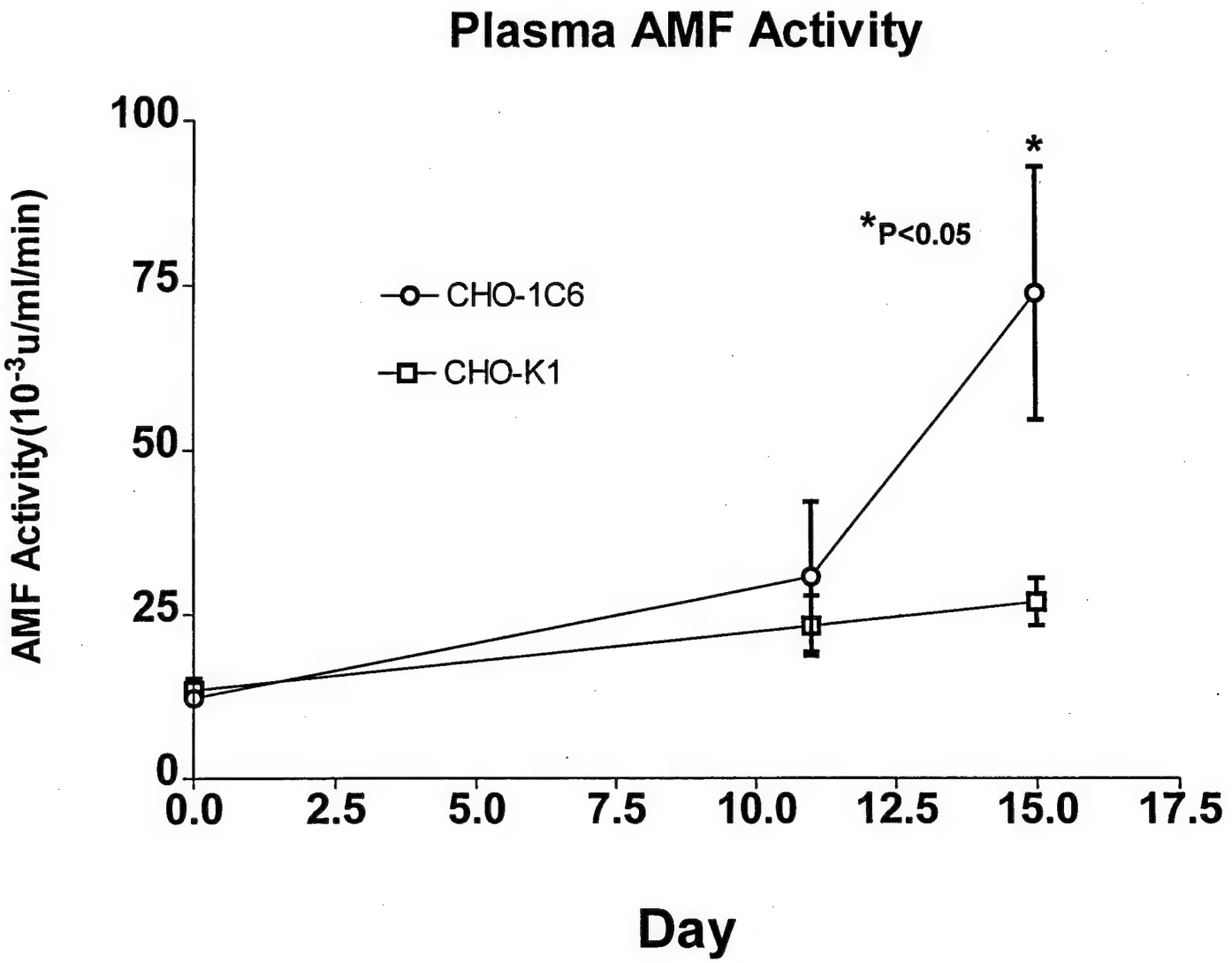

Figure 10. Plasma AMF activities were determined from retroorbital blood samples obtained at baseline and sacrifice. Whole blood ionized Calcium concentrations were unchanged.

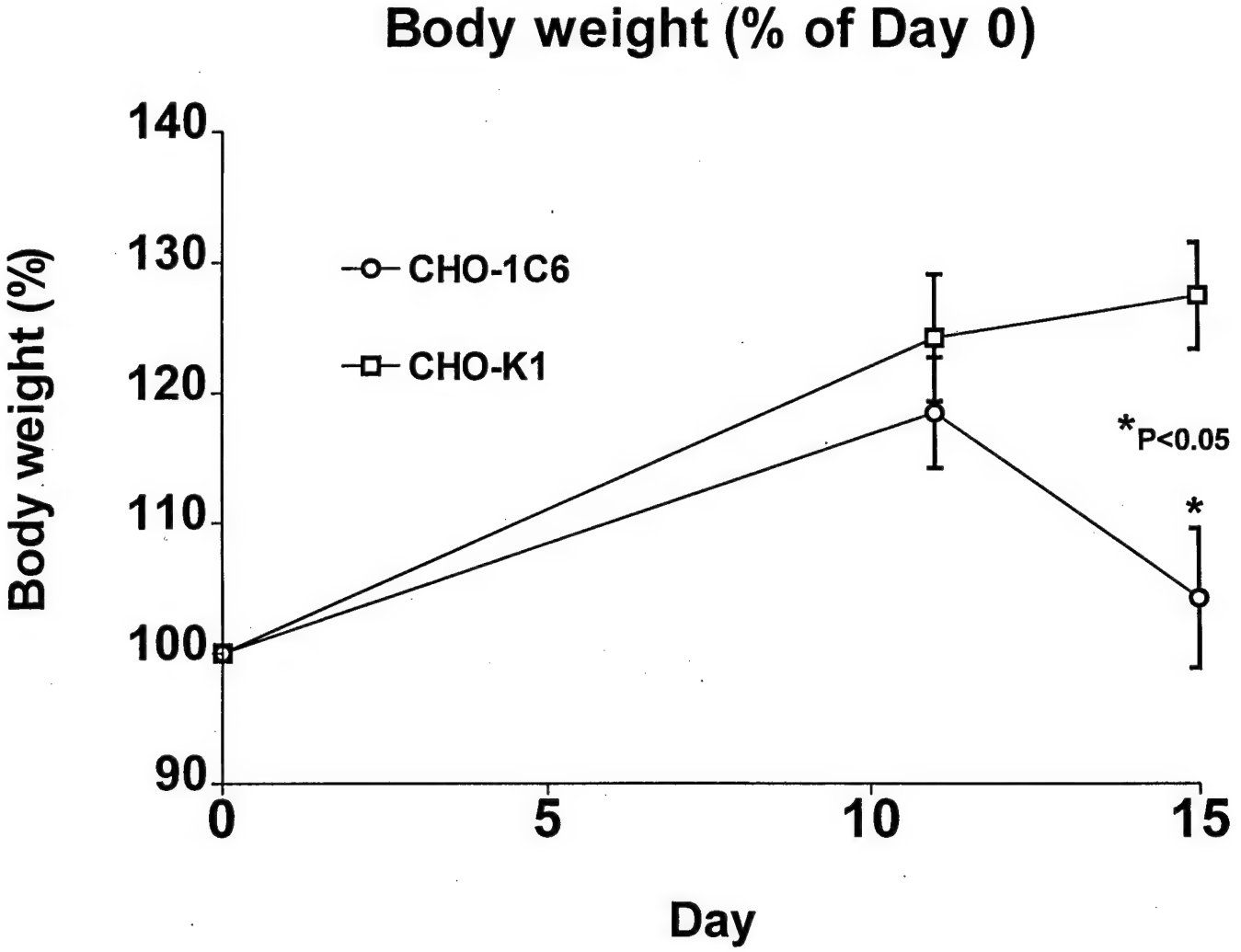

Figure 11. Animals (5/group) were weighed at days 0, 11, and 15.

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